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SEPARATION AND PARTIAL PURIFICATION OF CENTRAL NERVOUS SYSTEM PEPTIDES FROM *LIMULUS POLYPHEMUS* WITH HYPERGLYCEMIC AND CHROMATOPHOROTROPIC ACTIVITY IN CRUSTACEANS [1, 2]

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Thirty-six years ago a substance in the central nervous system (CNS) of the chelicerate arthropod *Limulus polyphemus* was shown to possess chromatophorotropic activity when tested on the mandibulate arthropod *Uca pugnax* (Brown and Cunningham, 1941). More recent studies have demonstrated that CNS extracts from *Limulus* are also chromatophorotropic in a variety of other decapods, including both brachyuran and natantian species (Fingerman, Bartell, and Krasnow, 1971; Herman and Dallmann, 1975). Other experiments have shown that arthropod molting hormones (ecydsones) are present and active in *Limulus* (Krishnakumaran and Schneiderman, 1970; Jegla, Costlow and Alspaugh, 1972; Winget and Herman, 1976). The existence of both CNS material with crustacean neurosecretory hormone activity and ecdysones in this species suggests that *Limulus* might also produce other substances with arthropod hormone activity. If so, neuroendocrinological studies of *Limulus* could be of major importance in attempts to understand the basic properties and evolution of arthropod neuroendocrine regulatory mechanisms. Against this background a series of studies were conducted testing the effects of CNS extracts from *Limulus* in known arthropod neurosecretory hormone bioassays. During this work the existence of a CNS substance causing hyperglycemia in the freshwater crayfish, *Orconectes immunis*, was discovered. Initial studies on this substance, and evidence demonstrating that it is not the above-mentioned chromatophorotropin, are presented below.

Materials and Methods

Adult specimens of *Limulus polyphemus*, obtained from the Marine Biological Laboratory, Woods Hole, Massachusetts, were maintained without feeding in Instant Ocean artificial sea water at 12° C. Specimens of *Orconectes immunis*, obtained from Trans-Mississippi Biological Supply, St. Paul, Minnesota, were maintained in dechlorinated tap water aquaria at 12° C and fed Gainesburger dog food three or four times a week. Specimens of *Uca pugilator*, supplied by Gulf Specimen Co., Panacea, Florida, were held in Instant Ocean sea water aquaria at 18° C and fed Gainesburger dog food weekly. Eyestalks were removed from specimens of *Orconectes* and *Uca* 24 hr prior to experiments.

Central nervous systems from *Limulus* were removed by ventral dissection,

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cleaned of adhering non-CNS material, weighed to the nearest mg and either immediately homogenized, or frozen on dry ice for lyophilization. Extracts were prepared with ethanol, acetone, 0.1 and 1.0 N acetic acid, and 0.1 M ammonia. The typical extraction protocol was as follows. The CNS was placed in a volume of solvent and thoroughly homogenized in a Potter Elvejem homogenizer at 4° C. The homogenate was then centrifuged for 15 min at 12,100 × *g* in a Sorval Superspeed RC2-B refrigerated centrifuge run at 4° C. The supernatant was saved, while the pellet was re-extracted three to four times to a total of 20 volumes. The pooled supernatants were then boiled for three min and recentrifuged as mentioned above. The crude extract was either used immediately or lyophilized for storage at −20° C. In most cases, the lyophilized residues were redissolved in distilled water at concentrations appropriate for injections or column chromatography. In some experiments crude extract was made 10^{-3} M with thiodiglycol (Sigma). Variations from the above procedure are cited in the text.

Assays of the CNS chromatophorotropin from *Limulus,* hereafter referred to as LUC, were conducted as previously described (Herman, 1975). In brief, the melanophore response of 5–10 eyestalkless female *Uca* to 10 μl aliquots of extract or solvent was observed, the control values subtracted from the experimental values, and the mean response per *Uca* calculated in chromatophore units.

Assays of CNS hyperglycemic activity from *Limulus* were performed on eyestalkless, mixed sex *Orconectes* randomly assigned to individual containers holding enough dechlorinated tap water to just cover the carapace. Injections of 50–100 μl of solvent or extract were made with disposable 1 ml tuberculin syringes fitted with 25 gauge needles. Hemolymph samples, withdrawn from the ventral abdominal or cephalothoracic sinus, were assayed for glucose by the Glucostat (Worthington Biochemicals) method (Meites, 1965) or for total carbohydrate by the Anthrone (Sigma) method (Chaykin, 1966). A Beckman DB-G spectrophotometer, set at 540 nm for Glucostat and 620 nm for Anthrone, was used for all colorimetric determinations. Glucose was used as the standard for both assay procedures.

Chromatography of LUC and experiments concerning both LUC and the hyperglycemic factor were conducted at 4° C on a Sephadex G-25 Fine (Pharmacia) column, 1.5 × 90 cm, equilibrated with 1.0 N acetic acid made 10^{-3} M with thiodiglycol. The flow rate was 25 ml/hr and 2.5 ml fractions were collected. Absorbancy of all fractions was read at 280 nm. The fractions were lyophilized and redissolved in distilled water. The column was calibrated with lysozyme (14,000), ACTH (4,570), glucagon (3,600) and bacitracin (1,400), all from Sigma.

The hyperglycemic factor was further chromatographed on a Sephadex G-50 Fine (Pharmacia) column, 2 × 40 cm, equilibrated with 0.1 N acetic acid made 10^{-3} M with thiodiglycol. This column was run at 4° C with a flow rate of 4.6 ml/hr, and fractions of 4.6 ml were collected. The fractions from this column were treated as previously mentioned with the exception that in some experiments the lyophilized fractions were redissolved in 0.1 N acetic acid. (Several experiments demonstrated that 50 μl of this solvent were not hyperglycemic in crayfish and thus did not interfere with the hyperglycemic assay.) This column was calibrated with bovine serum albumin (68,000), chymotrypsinogen (25,000), lysozyme (14,000) and glucagon (3600), all from Sigma.

LUC and the hyperglycemic factor were tested for susceptibility to some or all

TABLE I

Effect of CNS extracts from Limulus *on* Orconectes *hemolymph glucose.*

Material injected	Hemolymph glucose (mg %)
CNS equivalents	
0.04 (8)	8.7 ± 2.3
0.08 (4)	12.1 ± 4.4
0.20 (17)	19.2 ± 2.2
Solvent control (14)	3.6 ± 0.4

N in parentheses; experimental duration = 1 hr.

of the following enzymes: pepsin, protease, trypsin, chymotrypsin, thermolysin, and lysozyme (all from Sigma).

For each enzyme, extracts were incubated in the appropriate medium (enzyme concentration = 20 mg/ml) for 16 hr at 37° C. The reactions were terminated by boiling the reaction mixture for 5 min, after which the reaction mixtures were centrifuged to remove denatured enzyme. Extract without enzyme and enzyme without extract controls were subjected to the same conditions. The following buffers were used (Shepard, 1975): pepsin, 0.1 N acetic acid (pH 2.8); protease, 0.02 M HEPES-KOH (pH 7.5) containing 0.1 M calcium chloride; trypsin, 0.05 M Tris (pH 8.2) containing 0.01 M calcium chloride; chymotrypsin, 0.08 M Tris (pH 7.8) containing 0.1 M calcium chloride; thermolysin, 0.5 M Tris (pH 8.5) containing 0.005 M calcium chloride; and lysozyme, 0.1 M phosphate, pH 7.0.

The data are reported as mean ± s.e.m. Some of these data were analyzed by Student's *t*-test; the term significance in this report refers to statistical significance in this test at the 5% level or better.

RESULTS

Effects of CNS extracts from Limulus *on* Orconectes *hemolymph carbohydrates*

Initial studies tested CNS extracts from *Limulus* for hyperglycemic activity in *Orconectes*. The results of a typical experiment, using acetone extracts of fresh *Limulus* CNS, are summarized in Table I. Significant elevations of hemolymph glucose were obtained with as little as 0.04 CNS equivalent/crayfish, and larger doses produced substantially higher reponses. Analysis of total hemolymph carbohydrate before and after injection yielded similar results; in one such experiment *Limulus* CNS extracts (0.20 CNS equivalent/animal) produced a 130.0 ± 17.3% increase in 10 crayfish, while injections of solvent or muscle extract into 20 animals elevated total carbohydrate by only 32.0 ± 7.5%. Comparable experiments have been performed several times over a period of 2 yr using horseshoe crabs and crayfish obtained in both summer and winter. These studies invariably demonstrated that *Limulus* CNS extracts contained material capable of rising *Orconectes* hemolymph total carbohydrate and glucose levels.

The above results are duplicated with acetic acid extracts of *Limulus* CNS; ethanol and ammonia extracts also cause significant responses, but hemolymph glucose increases are quantitatively less impressive. Hyperglycemic activity can

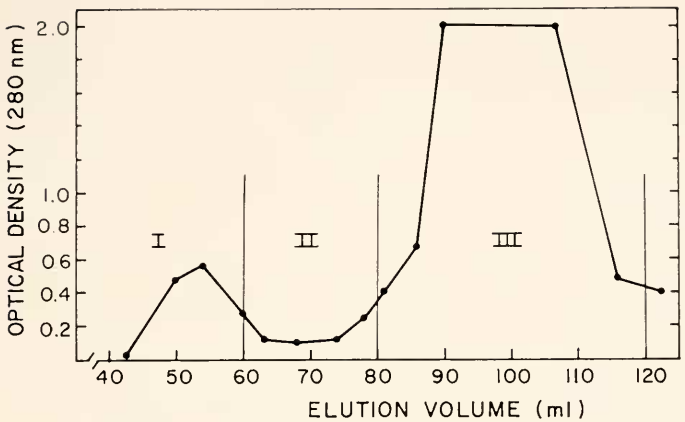

FIGURE 1. Chromatography of crude CNS acetic acid extracts from *Limulus* on Sephadex G-25 (fine). The extracts consisted of 3500 mg of CNS (wet weight) extracted as described and concentrated to 3 ml. The column was equilibrated with 1.0 N acetic acid; flow rate, 25 ml/hr; fraction volume, 4 ml; total volume, 130 ml; void volume, 54 ml. The absorbancy of each fraction was read at 280 nm (solid line). The fractions were pooled and assayed for hyperglycemic and melanophore dispersing activity (Table II).

usually be extracted from fresh or lyophilized CNS, but acetone extracts of lyophilized material have no effect. For convenience we have designated the active material in CNS extract LHGF, for *Limulus* hyperglycemic factor.

Separation of LHGF and LUC on Sephadex G-25

Preliminary gel filtration experiments (Fingerman *et al.*, 1971) indicated that LUC would elute in the last half of the elution volume on a Sephadex G-25 column. We therefore decided to attempt to separate LHGF and LUC by means of such a column. Concentrated *Limulus* CNS acetic acid extract was applied to the column, and 4 ml fractions were collected, pooled (as indicated in Fig. 1), lyophilized, and redissolved in distilled water for bioassay. The results were obvious (see Table II); LHGF, but not LUC, was present in Fraction I, which corresponded to the void volume. Neither activity was present in Fraction II, while Fraction III exhibited only LUC activity. From these experiments it was concluded that LHGF is excluded from Sephadex G-25 columns.

Preliminary characterization of LHGF

The preceding results clearly indicated that LHGF and LUC were separate substances. It was therefore decided to further characterize both LHGF and LUC to demonstrate their nonidentity.

Experiments were undertaken to determine the stability of LHGF. It was observed that unboiled acetic acid extracts of *Limulus* CNS are unstable at room temperature, with the majority of the hyperglycemic activity lost within 3 hr. This loss in activity could be prevented by brief boiling of the crude extract, or by storing the crude extract of 0° C. In addition, treatment of the crude extract with hydrogen peroxide (final concentration = 1%) reduced hyperglycemic activity by

TABLE II

Effects of Sephadex G-25 fractions of CNS extracts from Limulus *in* Orconectes *and* Uca.

Fraction tested	*Uca* response*	*Orconectes* response**
I	3.0 ± 1.8 (10)	86.0 ± 13.0 (10)
II	1.6 ± 1.1 (10)	17.0 ± 6.0 (10)
III	23.0 ± 3.0 (10)	22.5 ± 3.0 (10)
Solvent controls	0.0 (10)	16.5 ± 4.5 (10)

* Mean net chromatophore response; 10 μl of pooled fraction injected.

** Percentage of increase in total carbohydrate 60 min after injection; 100 μl of pooled fraction injected.

about one-third in 1 hr. On the basis of the above findings we now routinely boil the crude extracts briefly, centrifuge, and add thiodiglycol to a final concentration of 10^{-3} M. In addition, all extractions and chromatographic separations are performed at 4° C.

The next concern was to estimate the molecular weight of the LHGF *via* gel filtration. Initial separation of LHGF and LUC on Sephadex G-25 clearly demonstrated that a larger grade Sephadex was required; G-50 was selected. The results

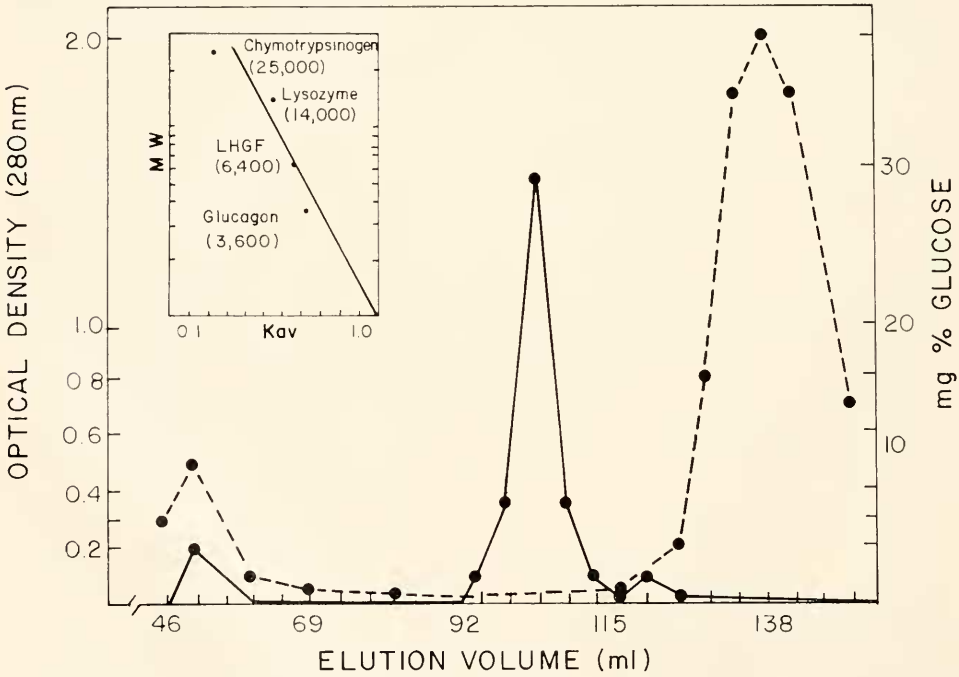

FIGURE 2. Chromatography of crude CNS acetic acid extracts from *Limulus* on Sephadex G-50 (fine). The column was equilibrated with 0.1 N acetic acid; flow rate, 4.6 ml/hr; fraction size, 4.6 ml; total volume, 140 ml; void volume, 51 ml. The absorbancy of each fraction was read at 280 (dashed line). The hyperglycemic activity of each fraction was tested on three animals per fraction (solid line). Insert shows G-50 calibration curve.

of a typical G-50 experiment are shown in Figure 2. On this column LHGF eluted as a single symmetric peak with an estimated molecular weight of 6400.

Active fractions from the G-50 runs were next treated with various proteolytic enzymes. Incubation of crude extracts with pepsin or protease resulted in a decrease in LHGF activity of 92.4% and 79.7%, respectively, while incubation with trypsin had no effect on activity.

Preliminary characterization of LUC

Several experiments were conducted on LUC to determine its stability, susceptibility to various proteolytic enzymes, and molecular weight. As reported elsewhere (Brown and Cunningham, 1941), brief boiling of crude extracts had no effect on activity. However, LUC did appear to be susceptible to oxidation. The combined results of experiments conducted at both 20° C and 37° C showed that untreated extracts held for 20 hr lost 32.9% of the original activity. In addition, treatment with thiodiglycol prevented this loss, while treatment with hydrogen peroxide lead to a 63.9% loss in activity. In view of these results, all subsequent extracts were

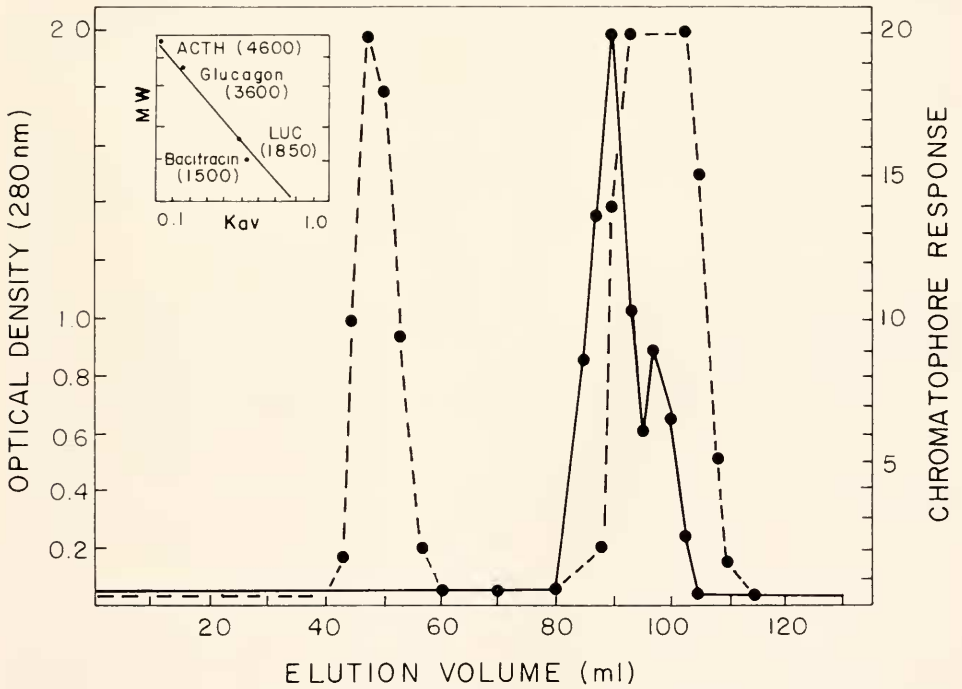

FIGURE 3. Chromatography of crude CNS acetic acid extract from *Limulus* on Sephadex G-25 (fine). The column was equilibrated with 1.0 *N* acetic acid; flow rate, 25 ml/hr; fraction volume, 2.5 ml; total volume, 134 ml; void volume, 46 ml. The crude extract consisted of 350 mg CNS (wet weight) extracted as described and concentrated to 2.5 ml. The absorbancy of each fraction was measured at 280 nm (dashed line). The chromatophorotropic activity of each fraction was tested on 18 animals/fraction and the response depicted (solid line). Insert shows G-25 calibration curve.

made 10^{-3} M with thiodiglycol. Treatment of crude *Limulus* CNS extracts with various enzymes, namely protease, pepsin, chymotrypsin, trypsin, and thermolysin resulted in a mean decrease in LUC activity of $92.8 \pm 1.4\%$, while the glycosidase lysozyme was without effect.

Molecular weight determination was done on a calibrated Sephadex G-25 column. As indicated in Figure 3, a major peak of activity eluted with an estimated molecular weight of 1,850 trailed by a secondary peak of activity. The presence of this latter peak, also noted by Fingerman *et al.* (1971), suggests the possible existence of more than one substance with LUC activity in the CNS of *Limulus*.

Discussion

These experiments have demonstrated the existence of two dissimilar substances with hormonal activity in crustaceans in *Limulus polyphemus* CNS extracts. One of these substances, the previously unreported LHGF, apparently has a molecular weight of about 6,400 daltons. In addition, it appears to be heat stable, inactivated by hydrogen peroxide, sensitive to some proteolytic enzymes, and unaffected by incubation with trypsin. These data collectively indicate that LHGF is a polypeptide. LHGF is clearly hyperglycemic in *Orconectes*, but lacking in melanophore pigment dispersing activity in *Uca*. The second substance(s) is the previously known chromatophorotropin, LUC. These studies have added to existing knowledge of this substance by demonstrating an apparent molecular weight of 1850 daltons, an estimate in agreement with earlier studies (Fingerman *et al.*, 1971). In addition, it has been shown for the first time that LUC is susceptible to a variety of proteolytic enzymes, including trypsin, and to the oxidizing agent hydrogen peroxide. Previous reports have demonstrated that LUC is heat stable (Brown and Cunningham, 1941; Herman, 1975). The total available data indicate that LUC is a peptide; it is chromatophorotropic in several crustaceans (see Herman and Dallman, 1975), but lacks hyperglycemic activity in *Orconectes*. On the basis of the above results, it can be concluded that *Limulus* CNS extracts contain at least two distinct peptides with different hormonal activity in crustaceans.

These data provide a basis for comparison of the properties of LHGF and LUC with those of known crustacean neurosecretory hormones. The available biological and chemical evidence indicates the existence of more than one decapod hyperglycemic hormone (Kleinholz and Keller, 1973; Kleinholz, 1976). Studies to determine the interspecific effect of various decapod hyperglycemic hormones (Keller, 1969) indicate little cross reactivity among the major suborders of decapods. However, partial chemical characterization of the hyperglycemic hormones from *Cancer magister, Pandalus jordani* and *Orconectes limosus* suggests chemical similarity; *i.e.*, all appear to have molecular weights of about 7,000 daltons, all are heat labile and susceptible to some proteolytic enzymes, and at least some appear to be resistant to trypsin and inactivated by hydrogen peroxide (Kleinholz, Kimball and McGarvey, 1967; Kleinholz and Keller, 1973; Kleinholz, 1976). LHGF also appears to fit into this basic scheme, with the notable exception that it is apparently heat stable. Decapod melanophore pigment dispersing hormones (MDH) currently seem to be less heterogeneous than the hyperglycemic hormones; they appear to be biologically similar, heat stable, susceptible to proteolytic enzymes and

oxidation, and to have molecular weights of about 2,000 daltons. The studies of Kleinholz (1976) suggest that the MDHs may possess a structure comparable to that of the distal retinal pigment hormone characterized by Fernlund (1976). The existing data therefore suggest that LUC and LHGF both resemble known crustacean hormones of comparable biological activity. Unfortunately, since only the distal retinal pigment hormone of *Pandalus borealis* has been totally characterized (Fernlund, 1976), the question of the identity, or nonidentity, of these various molecules will not be resolved until more complete structural data are available.

While the activity of LHGF and LUC in decapods is evident, the role of these peptides in *Limulus* remains enigmatic. LUC cannot act on integumentary chromatophores in the horseshoe crab, since this species lacks such chromatophores. Similarly, we have been unable in several attempts to demonstrate a hyperglycemic effect of partially purified LHGF in *Limulus*.

It is becoming more evident that the neuroendocrine system of *Limulus* is deserving of further study. This species produces and uses ecdysones (Jegla *et al.*, 1972; Winget and Herman, 1976), and it possesses at least two neurosecretory hormone-like peptides active in mandibulates. It is certainly reasonable to expect that further studies on this species will be of major importance in our attempts to understand the neuroendocrinology of chelicerate arthropods and the evolution of arthropod neuroendocrine systems.

We wish to express our appreciation to William Sparkes and Louisa Moore for their contributions to this research.

Summary

1. Crude extracts of Limulus CNS cause hyperglycemia in *Orconectes immunis* and expand chromatophores in *Uca pugilator*.

2. The hyperglycemic action is due to a previously unknown polypeptide (LHGF) with an estimated molecular weight of 6400 daltons. LHGF is inactivated by hydrogen peroxide, pepsin, and protease, but unaffected by trypsin and brief boiling.

3. The chromatophorotropic activity is due to the previously reported substance, LUC. LUC is shown to be a peptide with an approximate molecular weight of 1850 daltons; it is inactivated by hydrogen peroxide, protease, pepsin, trypsin, chymotrypsin, and thermolysin.

4. LUC and LHGF activity can be readily separated by gel filtration on a Sephadex G-25 column.

5. The similarity of LUC and LHGF to known crustacean hormones is discussed.

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